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Inhibition of hardy kiwifruit (*Actinidia aruguta*) ripening by 1-methylcyclopropene during cold storage and anticancer properties of the fruit extract

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ABSTRACT

Hardy kiwifruits (*Actinidia arguta*) were treated with 20 μl/l 1-methylcyclopropene (1-MCP) for 16 h at 10 °C and subsequently stored at 1 ± 0.5 °C. Anticancer properties of the fruit extracts were tested against five different human cancer cells. The hardy kiwifruits, without 1-MCP treatment, showed increases in both respiration and ethylene production rates during fruit storage. The 1-MCP treatment remarkably inhibited fruit ripening by reducing respiration and ethylene production. Fruits with the 1-MCP treatment could be stored for up to 5 weeks by maintaining higher fruit firmness, ascorbic acid and total phenolic contents compared to the control. The hardy kiwifruit extracts showed anti-proliferative effects to Hep3B and HeLa cells but not to HT29, HepG2 and LoVo cells. These results suggest that the application of 1-MCP at harvest effectively delayed the ripening process of the fruits, and the fruit extract had beneficial effects for the prevention of human cancer growth.

1. Introduction

Actinidia aruguta (Seib. Et Zucc.) Planch. Ex Miq., known as 'hardy kiwifruit' or 'baby kiwifruit' in English or 'Darae' in Korean, has recently become popular in the market because consumers want fruits with new tastes and healthy food. The hardy kiwifruits are smaller than fuzzy kiwifruits (*Actinidia deliciosa*) and can be eaten whole, without peeling (Krupa, Latocha, & Liwińska, 2011). The plants of hardy kiwifruit commonly exist in mountainous areas and are commercially cultivated in countries with colder climates, such as Japan, China and New Zealand along with some European countries, including Poland (Krupa et al., 2011; Ohashi, 1989; Okamoto & Goto, 2005). New cultivars of hardy kiwifruits, such as 'Cheongsan', 'Saehan', 'Daesung' and 'Chilbo', have been developed by breeding programs, and their cultivation areas have increased, mainly in the Kangwon-do area (NE areas of South Korea with mountainous terrain). To overcome their small sizes (~10 g), studies to increase fruit size, by selecting hexaploid cultivars (Okuyama, 2000) or by applying N_1-(2-chloro-4-pyridyl)-N_3-phenylurea at different growth stages of the crop (Kim et al., 2006), have been conducted.

Hardy kiwifruits are harvested when sugar levels reach 10–14 °Brix. If the fruits are left on the vine, the fruit will reach 18–25 °Brix (Strik, 2005). Although the quality is best when the fruits are ripened on the vine to maximize the development of aroma and flavor, their shelf life is then shortened. Therefore, hardy kiwifruits are commonly picked before they are vine ripened; otherwise, they would be too soft for package and shipping to commercial markets (Strik & Hummer, 2006). The storage life of hardy kiwifruits, picked at the firm and mature stage, is only 1–2 months at 0 °C (Strik & Hummer, 2006). In commercial markets in Korea, the recommended storage period of hardy kiwifruits is one to 2 weeks and an additional 2 or 3 days for shelf life. The main reasons for the short storage life are fruit softening, skin wrinkling due to water loss (dryness) and fruit decay. Fruit softening rapidly increases at the room-temperature ripening period, after harvest or cold storage (Krupa et al., 2011). Unlike *A. deliciosa*, the hardy kiwifruits are very sensitive to dryness because of their smooth peels that lack hair. This phenotype characteristic is the main reason for the short-storage time and fast loss of postharvest quality (Strik, 2005).

Fisk, Silver, Strik, and Zhao (2008) extended the storage life of hardy kiwifruits, by using edible coating materials consisting of mixtures of various formulas, such as calcium caseinate, chitosan, PrimaFresh 50-V and Semperfresh. Krupa et al. (2011) reported that hardy kiwifruits stored in common cold storage gradually lost physicochemical quality over 4 weeks due to decreases in ascorbic

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acid and phenolic compounds. Contrary to the fruit of *A. deliciosa*, which can be stored in cool conditions for up to 5 months, the storage time of hardy kiwifruits is usually no longer than 10–12 weeks and varies from year to year (Strik, 2005). Although several studies on the ripening physiology and optimum postharvest technology of hardy kiwifruits have been reported, the available information is still limited.

Ethylene is a ripening hormone in climacteric fruits. Its biosynthetic process is complex, and the major steps were elucidated by Yang and Hoffman (1984). Briefly, ethylene is biosynthesized from the precursor methionin (MET). The conversion of MET to *S*-adenosylmethionine (SAM) is mediated by SAM synthase, and SAM is catalyzed to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). ACC oxidase (ACO) converts ACC to ethylene. Lipoxygenase (LOX) is also associated with the ripening and senescence of kiwifruit (Zhang et al., 2006). Degradation products from the LOX pathway induce cellular oxidative stress during fruit ripening (Rogiers, Kumar, & Knowles, 1998). Specific LOX gene expressions, that induce fruit ripening and aroma production in kiwifruits, are up-regulated by ethylene (Zhang et al., 2009).

Thus, the use of an ethylene biosynthesis inhibitor or action inhibitor would be beneficial to delay fruit ripening. 1-Methylcyclopropene (1-MCP), which is an ethylene action inhibitor (Sisler & Serek, 1997), has been widely used as a postharvest treatment to control both fruit ripening and softening for a wide range of fruits. The application of 1-MCP to intact or fresh-cut kiwifruit significantly inhibited fruit softening (Antunes, Dandlen, Cavaco, & Miguel, 2010; Boquete, Trinchero, Fraschina, Vilella, & Sozzi, 2004; Deng, Jiang, Mu, Wanf, & Wang, 2013; Koukounaras & Sfakiotakis, 2007). The ripening of 'Hayward' kiwifruits treated with 1-MCP after cold storage was significantly delayed due to low ethylene production, as well as the inhibition of cell wall degradation enzyme activity (Boquete et al., 2004). During shelf life after the cold storage of 'Qinmei' kiwifruits harvested at higher ripening stages, 1-MCP reduced the decay incidence. However, 1-MCP-treated fruits had a lower consumer acceptance due to a reduced eating quality compared to untreated fruits (Deng et al., 2013).

Hardy kiwifruits contain high amounts of ascorbic acid (25–155 mg/100 g fresh weight) (Kabaluk, Kempler, & Toivonen, 1997) and are relatively high in many nutraceuticals. Gallic acid, a major phenolic compound of the fruits, is present at a concentration of approximately 2 mg/g fresh weight. Total antioxidant activity ranges from 1.6 to 2.3 ascorbic acid equivalents/g fresh weight (Fisk, Mcdaniel, Strik, & Zhao, 2006). The beneficial health effects of hardy kiwifruit extracts have been reported, particularly regarding dermatological uses of the fruit extracts. The reports have shown that the natural immune modulator system derived from the fruit extract has a therapeutic effect on atopic dermatitis (Chen et al., 2006; Kim, Lee, Son, & Kim, 2009; Park et al., 2005, 2007). The extract obtained from dried *A. aruguta*, named DA-9102, was a natural medicine candidate to control atopic dermatitis in Korea (Choi et al., 2008).

The objectives of this research were to examine effects of 1-MCP on the postharvest quality changes of hardy kiwifruits, to extend the storage life of the fruits, and to explore the therapeutic effects of the extract against various human cancer cell lines. We further studied how the ripening-related gene expressions of hardy kiwifruits were affected by 1-MCP during cold storage.

2. Materials and methods

2.1. Plant material

Hardy kiwifruits cv 'Cheongsan', which were bred in Korea in 2005, were grown in the county of Yeongwol, Gangwon-do, Korea. Fruits were harvested on 29 August, 2013, 85 days after flowering, at the firm and mature commercial harvest stage. The characteristics of the fruits at harvest were green skin color, 15–17 N of firmness, and 7–8 °Brix of soluble solids content. They were selected for uniform size, appearance, and lack of defects and stored at 1 ± 0.5 °C after 1-MCP treatment (described below).

2.2. 1-MCP treatment, cold storage and sampling

Hardy kiwifruits were randomly divided into four lots (15 kg/a lot), and each lot was kept in a 50-l plastic container. Two lots were exposed to 20 μl/l 1-MCP for 16 h at 10 °C. 1-MCP was released by adding a buffer agent to calculated amounts of Smart Fresh powder (Agrofresh Rohm and Hass, Philadelphia, USA) according to the manufacturer's instruction. The other two lots were kept under the same experimental conditions without 1-MCP. After treatments with or without 1-MCP, each lot was again subdivided into 15 groups (1 kg/group) and placed in a plastic tray (20 × 10 × 5 cm) without a cover. They were stored at 1 ± 0.5 °C in ambient air with a relative humidity of 75–80% to simulate commercial cold storage. As replicates for each treatment, three plastic trays were analyzed weekly for quality attributes during cold storage. For gene expression analysis, fruits were sampled at zero, three and 5 weeks. To test the anticancer properties of the fruit extracts, harvested fruits were immediately frozen at −80 °C, freeze-dried, and powdered.

2.3. Measurement of fruit weight loss and firmness

Weight loss was determined as the percent of weight loss per initial fruit weight. The fruit firmness values of ten randomly selected fruits per replicate were measured using a texture analyzer (TA.XT2, Stable Micro Systems, Scarsdale, NY, USA) fitted with 2-mm flat probe. Each fruit was compressed 2 mm at a rate of 0.5 mm/s, and the maximum force developed during the test was recorded and expressed in Newton (N).

2.4. Measurement of carbon dioxide and ethylene production

Randomly selected fruits (approximately 200 g) from each replicate were placed in a 1-l sealed jar, with a septum in the lid, for sampling headspace gas. The fruits were allowed to equilibrate at room temperature for 2 h, and the jars were closed and kept for 3 h. A 1-ml sample of headspace gas was removed from each jar using a gastight syringe. Carbon dioxide and ethylene production were measured by a gas chromatography (GC) system (YL6500, YoungLin, Anyang, Korea) equipped with a thermal conductivity detector for carbon dioxide and a flame ionization detector for ethylene. Carbon dioxide was analyzed using a porapak column (1.5 m × 6 mm). Ethylene was analyzed using an alumina column (1.5 m × 6 mm). The oven, injector and detector temperatures were set at 70, 110 and 150 °C for carbon dioxide and 70, 110 and 250 °C for ethylene analyses, respectively.

2.5. Measurement of ascorbic acid content

Ten fruits were randomly selected from each replicate and macerated for 1 min until the tissues were completely homogenized using a home mixer. A 3-g slurry sample from the homogenates was mixed with 30 ml of 3% meta-phosphoric acid in water and homogenized. The homogenate was filtered with a filter paper (Whatman No. 1) and a 20-μl sample was injected into the HPLC system (YL9100, YoungLin, Anyang, Korea), which consisted of a binary pump, an autosampler, and a diode array detector. A μBondapak NH_2 column (Waters, 3.9 × 30 mm, 10 μm) with a guard column was used, and the solvent was acetonitrile:water

Table 1
Primer sequences used for RT-PCR and RT-qPCR analyses of *AcACS*, *AcACO*, *AcLOX*, and *AcACT* in *Actinidia aruguta* 'Cheongsan'.

Gene	Accession number	Forward primer sequence (5′ → 3′)	Reversed primer sequence (3′ → 5′)
AcACS	AB007449	CAACCTCCTGCTCACGTTCA	GTTGGAGTATATGGCCCCGA
AcACO	JQ062390	TGCTTGTGAGAACTGGGGCTT	GCGCAAGAAGAAGGTGCTTTC
AcLOX	AB300613	CATGCAGTAATCGAGCCATTC	CAGCCGGGAGTGCCTGCTCTG
AcACT	DQ682826	ACCTTGCTGGCCGTGATCTA	AGCTCCAATTGTGATGACCTGA

(7:3, v/v) with 0.01 M ammonium dihydrogen phosphate at a flow rate of 1 ml/min. Ascorbic acid was detected at 255 nm. Standard curves were constructed between 0 and 500 μg/ml of ascorbic acid and used to quantify the samples.

2.6. Measurement of total phenolic content

A 3-g slurry sample, as prepared above, was homogenized with 30 ml of 100% methanol, and total phenolic contents were determined with the Folin–Ciocalteu reagent using gallic acid as a standard (Slinkard & Singleton, 1977). Absorption was measured at 765 nm, and the results were expressed as mg gallic acid equivalence (GAE)/100 g of fresh weight.

2.7. Extraction of total RNA

Total RNA was isolated following the method described by Gambino, Perrone, and Gribaudo (2008). CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2.5% PVP, and 2% of β-mercaptoethanol at pH 8.0) was pre-heated at 65 °C. Five fruits were randomly selected from each replicate, and each fruit was horizontally sliced to a 5-mm thickness. The sliced fruit was immediately chilled with liquid nitrogen, placed in a plastic bag, and kept at −80 °C before RNA analysis. A 5-g sliced sample from each bag was ground to a fine powder in liquid nitrogen. The powdered sample was then transferred to 50-ml polypropylene tube containing 15 ml of CTAB extraction buffer. The tube was vortexed and then incubated at 65 °C for 10 min. Fifteen milliliters of chloroform:isoamyl alcohol (24:1 v/v) was added to the tube; the sample was then vortexed again and centrifuged at 11,000×*g* for 10 min at 4 °C. The supernatant was transferred to new 50-ml tube, and LiCl (3 M at final concentration) was added. The mixture was incubated at 4 °C for 30 min and then centrifuged at 21,000×*g* for 20 min at 4 °C. The pellet was resuspended with 0.5 ml of SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, 1 mM EDTA, at pH 8.0), pre-heated to 65 °C and transferred to a micro-centrifuge tube. Five hundred microliters of chloroform:isoamyl alcohol (24:1, v/v) was added to the tube, and the sample was vortexed and centrifuged at 11,000×*g* for 10 min at 4 °C. The supernatant was transferred to new micro-centrifuge tube, and 0.7 ml of cold isopropanol was added followed by centrifuging at 21,000×*g* for 15 min at 4 °C. The pellet was mixed with 50 μl of 70% ethyl alcohol and again centrifuged at 21,000×*g* for 5 min at 4 °C. Total RNA was briefly dried and re-suspended with 100 μl of DEPC-water.

2.8. RT-PCR and RT-qPCR analysis

For RT-PCR and RT-qPCR analysis, the first strand cDNA was synthesized using the *amfiRivert* Platinum cDNA Synthesis Master Mix (GenDEPOT, Texas, USA) from 5 μg of total RNA. Both PCRs were performed using candidate primers including *AcACS*, *AcACO*, *AcLOX*, and *AcACT* (Table 1) with a template of a 1:10 diluted solution of the first strand cDNA product. Candidate primer sequences are available in GenBank from the National Center for Biotechnology Information.

RT-PCR was carried out in 20-μl reaction volume, equipped with a LifeECO thermal cycler (Hangzhou Bioer Technology Co., Hangzhou, China). The PCR reaction mixture consisted of 5 μl cDNA, 0.2 mM dNTP, 10 pM of each primer, 40 mM Tris-HCl at pH 9.0, 120 mM KCl, 6 mM $MgCl_2$, and 0.5 units of Solg™ Taq polymerase (SolGent, Daejeon, Korea). The thermal cycling parameters were 94 °C for 4 min, 32 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 30 s, and 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1.2% agarose gels containing Ecodyne reagent (Dyne bioscience, Korea).

RT-qPCR was carried out in a 10-μl reaction volume equipped with CFX Connet (Bio-Rad, California, CA, USA). The PCR reaction mixture contained 2 μl cDNA, 4 pM of each primer, and 5 μl of 2 × Labopass SYBR Green Q Master (Cosmogenetech, Seoul, Korea). The thermal cycling parameters were 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, 95 °C for 10 s, and a melting curve of 65–95 °C at an increment of 0.5 °C in 10 s. Each reaction was performed in triplicate, and no-template controls for each primer pair were included in each run. The relative intensities of samples at week 0 were set to a value of 1.

2.9. Fruit extraction using a Soxhlet-type apparatus

Powdered fruits (500 g) were extracted using a Soxhlet apparatus with 1.3 l of methanol:water (8:2, v/v) at 55–65 °C for 16 h. The extract was filtered through Whatman filter paper (No. 1) to remove any particles. The extracts were completely concentrated using a rotary evaporator (NN series, EYELA, Tokyo, Japan) in a water bath at 40 °C. The fruit concentrate was freeze-dried and kept at −20 °C for anticancer property testing.

2.10. Cell culture and MTT assay

We tested five types of human cancer cells, such as HT29, HepG2, Hep3B, HeLa and LoVo. Cell culture and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay were carried out by the method described by Lee et al. (2014). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in a 5% CO_2-humified incubator at 37 °C.

The toxicity of the fruit extracts to cancer cells was measured by the MTT assay. Each cell line was plated at a density of 0.8×10^4 cells per well in 200 μl of DMEM medium and incubated for 24 h to stabilize. The growth medium was removed, and the media containing increased fruit extract concentrations ranging from 0 to 1.0 μg/ml were added to the cells and incubated for 48 h. The fruit extracts were further dissolved in dimethyl sulfoxide (DMSO) prior to diluting the stock solution in culture media. The highest concentration of DMSO was below 0.1% in all tests. After 4 h of incubation with thiazolyl blue tetrazolium bromide dissolved in sodium perborate (5 mg/ml), the media, MTT reagents, and dead cells were aspirated. One hundred microliters of DMSO was added to all wells and mixed thoroughly for 2 h to dissolve the purple formazan crystals. After ensuring that all crystals were dissolved, the MTT absorbance was measured at 570 nm using a multi-detection microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA).

2.11. Statistical analysis

The experiments were designed to be completely randomized. All experimental analyses were performed in triplicate, and means and standard deviations were calculated. For the MTT assay, eight replicates were performed for each cell line, and means and standard deviations were expressed. The figures were generated using Sigma Plot 10.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Fruit quality during cold storage

The ethylene action inhibitor 1-MCP effectively inhibited the ripening of hardy kiwifruits during cold storage. The overall quality of 1-MCP-treated fruits was maintained for up to 5 weeks, as determined by the overall fruit quality and nutritional values (Fig. 1). The appearances of the hardy kiwifruits stored at 0 °C for 5 weeks are shown in Fig. 2. After 5 weeks, weight loss increased by over 30% in the control and 20% in the 1-MCP-treated fruits (Fig. 1A). A great difference in fruit firmness was observed between fruits with and without 1-MCP (Fig. 1B). The hardy kiwifruits without 1-MCP treatment exhibited a sharp decline in fruit firmness after 1 week and became over-ripened during the storage period; thus, the fruit firmness of the control could not be measured after 5 weeks. However, fruits treated with 1-MCP showed a relatively high firmness of over 12 N during the entire storage period. Similar results have been reported in hardy kiwifruit 'Huan optimal No. 1', and 1-MCP extended the period to reach fruit softening in a concentration-dependent manner (Wang, Xu, Feng, & MacArthur, 2015).

The critical factor responsible for reducing the quality of harvested hardy kiwifruits is fast softening due to water loss and fruit decay in the commercial market place. In this study, fruit decay was negligible, and a total of 2–3% of the fruits exhibited a fungal infection during cold storage (data not shown). In the control, 30% weight loss occurred after 5 weeks of storage under 75–80% relative humidity (Fig. 1A). However, the loss could be reduced to less than 30% if the relative humidity was maintained at higher than 90%. As shown in Fig. 1B, fruit ripening and senescence, which resulted in fruit softening, occurred very quickly even though the hardy kiwifruits were stored in cold conditions. Therefore, the application of 1-MCP at harvest time is a very effective way to delay fruit ripening.

Hardy kiwifruit 'Cheongsan' had a relatively high ascorbic acid content of ~147 mg/100 g fresh weight at harvest (Fig. 1C). Although ascorbic acid decreased gradually with storage period in both the control and the fruit treated with 1-MCP, 1-MCP inhibited the degradation of ascorbic acid, helping maintain fruit freshness and delay fruit ripening (Fig. 1A and B). In contrast to the decrease in ascorbic acid content, the total phenolic contents were stable for 3 weeks in all experiments. In the control, total phenolic content decreased sharply after 3 weeks (Fig. 1D); the ascorbic acid and total phenolic contents of the control were 16% and 13% lower, respectively, at 5 weeks compared to at harvest.

Hardy kiwifruits are sometimes called a healthy fruit because they have high ascorbic acid, lutein and phenolic contents (Latocha, Krupa, Wolosiak, Worobiej, & Wilczak, 2012; Latocha, Wolosiak, Worobiej, & Krupa, 2013). Their nutraceutical content, which is a source of ascorbic acid, is 25–155 mg/100 g fresh weight depending on the cultivar (Kabaluk et al., 1997). Harvest maturity and storage conditions influence the physicochemical quality of hardy kiwifruits. Based on the acceptable fruit quality and nutritional value, fruits harvested at commercial maturity can be stored for 4 weeks under commercial cold storage conditions (Krupa et al., 2011). The ascorbic acid and phenolic contents of hardy kiwifruits depends on clones and either decrease during storage or remain unchanged (Krupa et al., 2011). Okamoto and Goto (2005) reported that the ascorbic acid contents of hardy kiwifruits did not change significantly after 2 months of storage at near 0 °C. However, these compounds generally decreased in fruits stored at cold temperatures for a longer period of time (Krupa et al., 2011), and in our study (Fig. 1C and D). Hardy kiwifruits 'Cheongsan' were

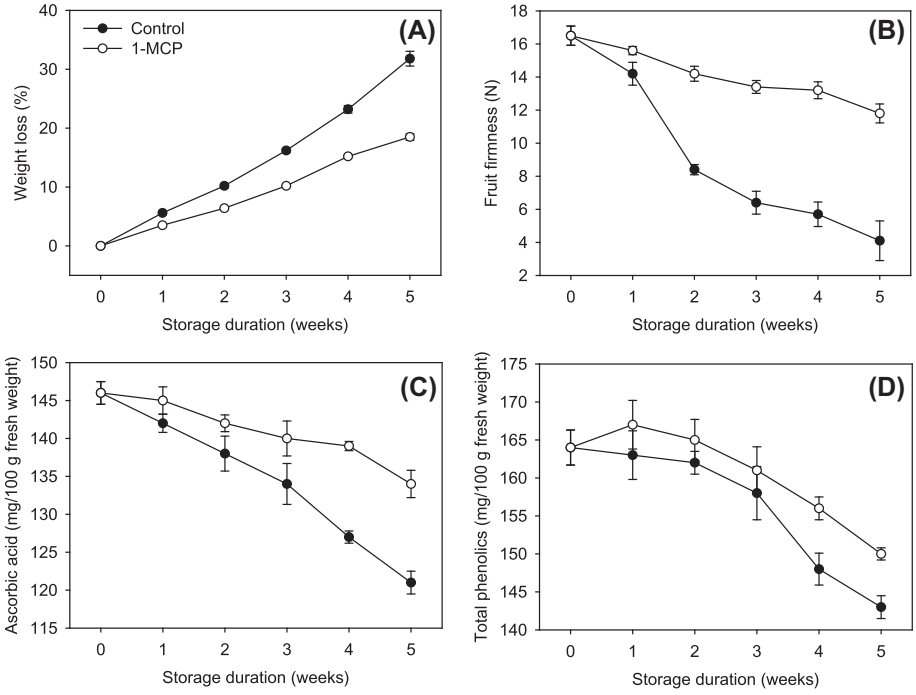

Fig. 1. Quality changes of hardy kiwifruits stored at 1 ± 0.5 °C for 5 weeks. Fruits were treated with 20 μl/l 1-MCP for 16 h at 10 °C and then stored. The fruit in the control was stored without 1-MCP treatment. Data were expressed as means ± standard deviations of three replicates.

Fig. 2. Appearances of hardy kiwifruits stored at 1 ± 0.5 °C for 5 weeks. Fruits were treated with 20 μl/l 1-MCP for 16 h at 10 °C and then stored. The fruit in the control was stored without 1-MCP treatment.

completely softened after 5 weeks and lost their marketable appearance due to over-ripening. 1-MCP effectively prolongs the fruit storage life for up to 5 weeks by delaying the softening and loss of nutritional value (Fig. 2).

3.2. Respiration rate and ethylene production

Hardy kiwifruit 'Cheongsan' showed high respiration and ethylene production rates in the control during cold storage (Fig. 3). Fruits without 1-MCP treatment showed gradually increasing carbon dioxide and ethylene production over 5 weeks. On the other hand, the 1-MCP treatment remarkably reduced the respiration and ethylene production in the fruits. Fruits treated with 1-MCP did not show any peak rises during the entire storage duration. In the control, however, the respiration increased for 2 weeks and declined gradually thereafter. As respiration increased notably, ethylene production increased accordingly; and was identical with the trend of fruit softening shown in Fig. 1B.

A. deliciosa showed a typical climacteric characteristic (Boquete et al., 2004). The climacteric fruit ripening was clearly related to the increase in respiration and ethylene production during the development stage (Pech, Bouzayen, & Latchè, 2008). *A. aruguta* is a type of climacteric fruit, and ethylene speeds up the fruit ripening and softening (Wang et al., 2015). Li, Xin, Zhang, and Liu (2014) also reported that hardy kiwifruit is a climacteric fruit, and the endogenous abscisic acid content is related with fruit ripening during cold storage as it influences respiration, ethylene production, and pectase and amylase activities. Hardy kiwifruit ripening and senescence were partially dependent on endogenous ethylene biosynthesis (Menniti, Gregori, & Donati, 2004). Hardy kiwifruit 'Cheongsan' showed a climacteric-like peak rise in respiration rate (Fig. 3A) and a linear increase in ethylene production (Fig. 3B) during cold storage in the control. The respiration and ethylene production peaks appeared almost concomitantly.

3.3. Ripening-related gene expression

We confirmed the role of ethylene and 1-MCP in the ripening of hardy kiwifruits. To understand the regulation of two ethylene biosynthesis genes (*AcACS* and *AcACO*) and another ripening-related gene *AcLOX* by 1-MCP, both RT-PCR (Fig. 4A) and RT-qPCR (Fig. 4B–D) were conducted. Transcripts of *AcACS*, *AcACO*, and *AcLOX* were expressed more in the control, but 1-MCP down-regulated their levels (Fig. 4A). These results were also clearly observed in the RT-qPCR analysis. The transcript levels of *AcACS*, *AcACO*, and *AcLOX* were increased more in the control, and *AcACS* and *AcLOX* reached maximum levels at 3 weeks (Fig. 4B–D). The transcript level of *AcACO* increased continuously in accordance with fruit softening in the control (Fig. 1B) and coincided with the ethylene production pattern shown in Fig. 3B. The 1-MCP-treated fruits showed less than half the expression levels of *AcACS*, *AcACO*, and *AcLOX* compared to the control at 3 weeks, with the exceptions of *AcACS* and *AcLOX* in the fifth week (Fig. 4).

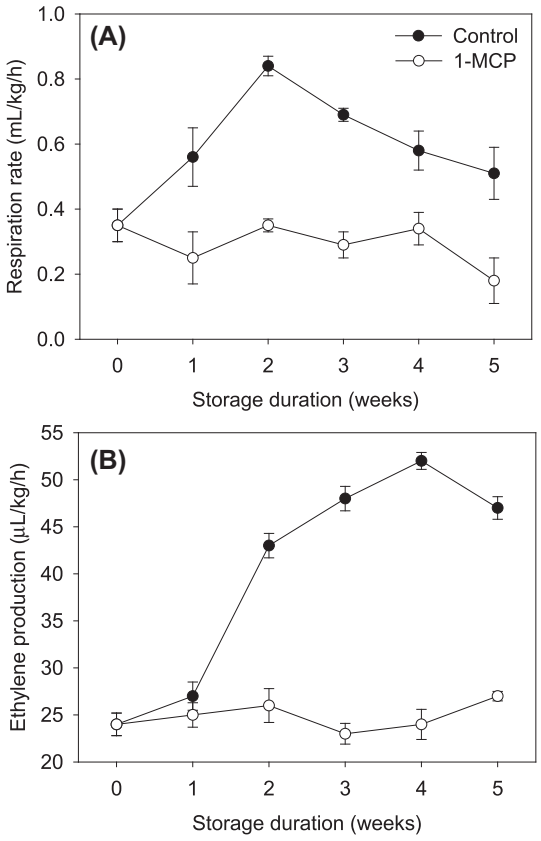

Fig. 3. Respiration rate and ethylene production of hardy kiwifruits stored at 1 ± 0.5 °C for 5 weeks. Fruits were treated with 20 μl/l 1-MCP for 16 h at 10 °C and then stored. The fruit in the control was stored without 1-MCP treatment. Data are expressed as means ± standard deviations of three replicates.

The above results showed that the transcript levels of the fruit ripening-related genes *AcACS*, *AcACO*, and *AcLOX* were strongly related to the ethylene production of hardy kiwifruit 'Cheongsan' during fruit ripening, and 1-MCP effectively inhibited the accumulation of their mRNA levels. The ethylene action inhibitor 1-MCP is well known to regulate genes encoding ethylene biosynthesis enzymes in climacteric fruits, such as 'Hayward' kiwifruits (Yin et al., 2009), 'Sanuki Gold' kiwifruits (Mworia et al., 2010) and 'Hakuho' peach fruits (Mathooko, Tsunashima, Owino, Kubo, & Inaba, 2001). The application of 1-MCP and its structural analogs 1-pentylcyclopropene and 1-octylcyclopropene to hardy kiwifruits, after harvest, postponed the appearance of respiration rate peaks and ethylene production peaks and delayed the softening and weight loss by suppressing ACS and ACO activities (Wang et al., 2015).

In general, LOX is closely associated with fruit ripening and qualities, such as aroma development (Zhang et al., 2009). Increased LOX activity promotes fruit softening by deriving degradation products from the LOX pathway, which contribute to the oxidative stress of cellular membranes during fruit ripening (Rogiers et al., 1998). Six different LOX genes were identified from kiwifruit and were regulated differently during fruit ripening and senescence. The expressions of *AdLOX1* and *AdLOX5* were up-regulated as fruits developed to the climacteric stage and by the presence of ethylene (Zhang et al., 2006). From our results shown in Fig. 4D, we confirmed that the expression of *AcLOX* was related to hardy kiwifruit ripening because it tended to increase with fruit ripening in the control, and 1-MCP highly down-regulated its expression.

The regulation of genes encoding the ethylene biosynthesis pathway or fruit ripening in hardy kiwifruit has not been reported yet. The results of this study suggested that the up-regulations of three ripening-related genes (*AcACS*, *AcACO*, and *AcLOX*) strongly affect hardy kiwifruit ripening by inducing fruit softening and ethylene production, as reported in other climacteric fruits, such

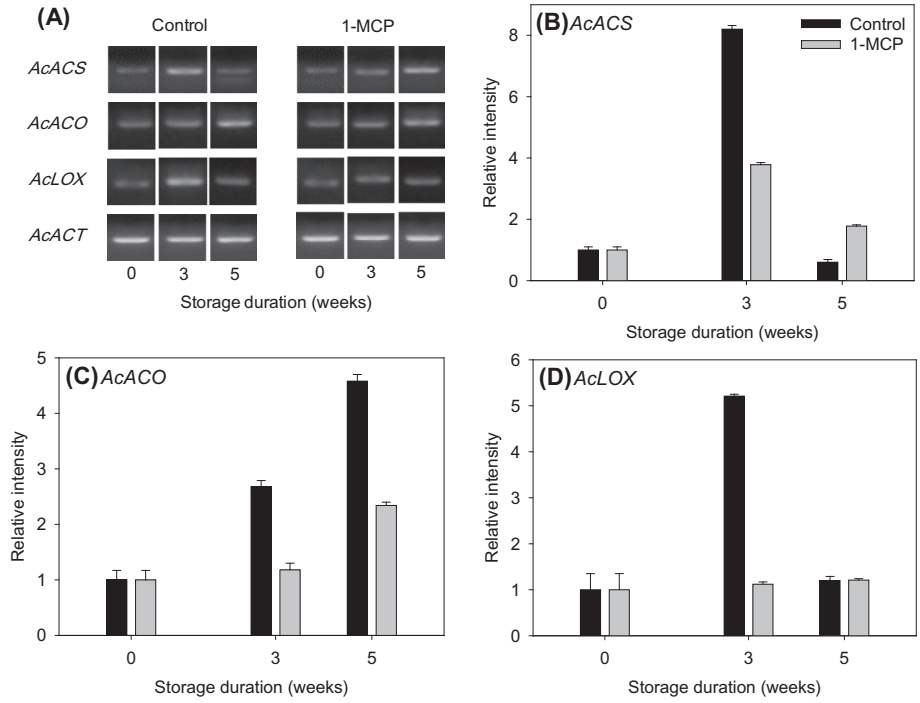

Fig. 4. RT-PCR and RT-qPCR analyses of ripening-related genes (*AcACS*, *AcACO*, and *AcLOX*) of hardy kiwifruits stored at 1 ± 0.5 °C for 5 weeks. Fruits were treated with 20 μl/l 1-MCP for 16 h at 10 °C and then stored. The fruit in the control was stored without 1-MCP treatment. Data are expressed as means ± standard deviations of three replicates.

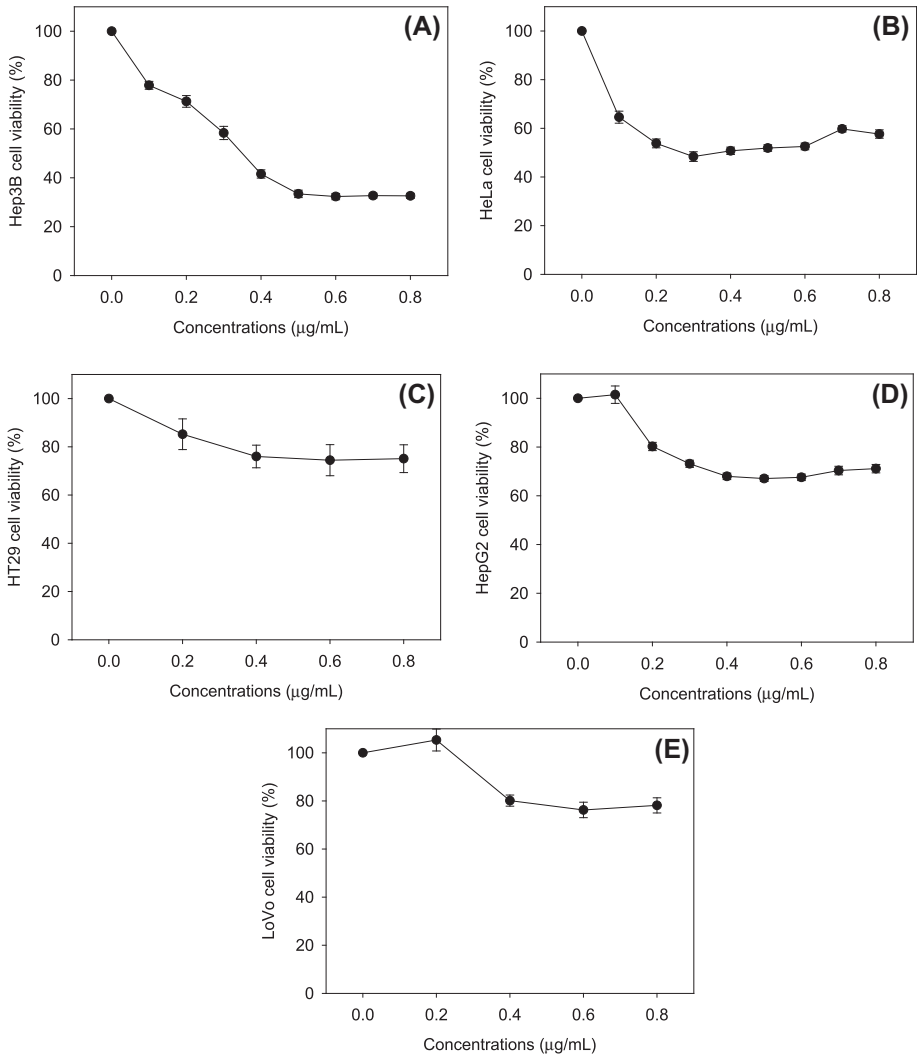

Fig. 5. Anticancer properties of the hardy kiwifruit extracts against five types of human cancer cells. The cells were exposed to various concentrations of the fruit extracts for 48 h at 37 °C. Data are expressed as means ± standard deviations of eight replicates.

as kiwifruit (Zhang et al., 2009) and tomato (Griffiths, Barry, Alpuche-Solis, & Grierson, 1999).

3.4. Anticancer properties of the fruit extracts

Hardy kiwifruit extracts exhibited different proliferative inhibitory effects against cancer cells (Fig. 5). The fruit extracts did not effectively inhibit the cell proliferation of HT29, HepG2, and LoVo; cell viabilities exceeded 60% regardless of concentration, which ranged from 0 to 0.8 μg/ml (Fig. 5C–E). However, the extracts exhibited dose-dependent inhibition of Hep3B. Hep3B showed the lowest cell viability of 32% at 0.6 μg/ml (Fig. 5A). The inhibition of HeLa cells by the extracts was also observed; the lowest cell viability of HeLa was 48% at 0.3 μg/ml (Fig. 5B). In the MTT assay, hardy kiwifruit extracts showed the most inhibition of Hep3B viability among the tested cancer cells, suggesting the proliferation inhibitory potential of the extracts. In addition to the clinical efficacy of *A. aruguta* extracts for atopic dermatitis (Kim et al., 2009) and inflammation (Kim, Hwang, & Park, 2014), we suggest possible roles of the fruit extracts for cancer inhibition, specifically against Hep3B cells.

Searching for new species that are rich sources of biologically active compounds has been the topic of many studies. The fruits and stems of *A. aruguta* have been used to relieve fever, thirst, jaundice and dropsy (Kim et al., 2014), and has become more popular in different countries. These beneficial effects resulted from the high nutritional values of ascorbic acid and phenolic compounds, which exhibit good antioxidant potentials (Latocha et al., 2012). To confirm these potential health benefits, further studies using various human cancer cells and individual purified compounds are necessary in both *in vitro* and *in vivo* systems.

4. Conclusions

We first conducted a postharvest investigation on the hardy kiwifruit 'Cheongsan' to understand the ripening physiology at the molecular level and suggested anticancer effects of the fruit extracts besides a well-known clinical effect in atopic dermatitis. The fruit showed climacteric-like characteristics after harvest, and 1-MCP treatment clearly delayed fruit ripening by down-regulating the expressions of *AcACO*, *AcACS*, and *AcLOX*, which are commonly known as ripening or senescence genes in fruits. We confirmed their putative ripening functions in hardy kiwifruits. 1-MCP extended the cold storage lives of the fruits for up to 5 weeks at 1 ± 0.5 °C by minimizing fruit softening and nutritional losses. The suggested health benefits produced by the hardy

kiwifruits might be helpful for selecting genetic resources for breeding programs as well as for the food processing industry to develop functional foods.

Conflict of interest

The authors declare no competing financial interest.

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